BIOLOGICAL COMPOSITIONS, COMPONENTS THEREOF AND USES THEREFOR

FIELD OF THE INVENTION

The present invention relates generally to an isolated Hepatitis B virus (HBV) with a surface component exhibiting an altered immunological profile relative to a reference HBV. A reference HBV is considered herein to comprise a composite or consensus nucleotide or amino acid sequence from HBV genotypes A through F. The isolated HBV of the present invention is considered herein to be a HBV variant relative to the reference HBV. The altered immunological profile renders the HBV variants of the present invention less susceptible to vaccines directed to the surface component. The HBV variants of the present invention generally arise from selective pressure following one or both of anti-HBV chemical therapy and in particular chemical therapy aimed at disrupting HBV polymerase activity or function and/or following immune pressure directed to the surface component. Immune pressure may result from natural exposure to HBV or following vaccination with an avirulent or attenuated HBV or with a component of an HBV. The present invention further provides a recombinant polypeptide and derivatives and chemical equivalents thereof corresponding to the surface component of the HBV variants. The HBV variants and recombinant polypeptides and their derivatives and chemical equivalents of the present invention are useful in biological compositions capable of inducing a neutralizing immune response to the HBV variant.

BACKGROUND OF THE INVENTION

Bibliographic details of the publications numerically referred to in this specification are collected at the end of the description.

The rapidly increasing sophistication of recombinant DNA technology is greatly

facilitating advances in the medical and allied health fields. This is particularly the case with the generation of recombinant vaccines and therapeutic compositions. Recombinant technology is providing the means to generate recombinant components of vaccines as well as providing genetic bases for screening or identifying useful components for therapeutic compositions.

Hepatitis B virus (HBV) can cause debilitating disease conditions ranging from subclinical infection to chronic active and fulminant hepatitis and can lead to acute liver failure.

The HBV genome comprises a series of overlapping genes in a circular, partially double-stranded DNA molecule (1) [see also Figure 1]. For example, the gene encoding DNA polymerase overlaps the viral envelope genes, Pre-S1, Pre-S2 and S and partially overlaps the X and core genes. The HBV envelope comprises small, middle and large HBV surface proteins. The large protein component is generally referred to as the HBV surface antigen (HBsAg) and is encoded by the S gene sequence. The Pre-S1 and Pre-S2 gene sequences encode the other envelope components (2).

The HBsAg comprises an antigenic region referred to as the "a" determinant (3). The "a" determinant is complex, conformational and dependent upon disulphide bonding among highly conserved cysteine residues. Genetic variation leading to changes in the "a" determinant has been implicated in mutants of HBV which "escape" the immunological response generated to conventional vaccines (4-8). One particularly common mutation is a glycine (G) to arginine (R) substitution at amino acid position 145 (G145R) of HBsAg. This mutation affects the "a" epitope region.

The increasing reliance on chemical and immunological intervention in treating or preventing HBV infection is resulting in greater selective pressure for the emergence of variants of HBV which are resistant to the interventionist therapy. Such variants are referred to as "escape" mutants.

There is a need to foreshadow potential vaccine escape variants of HBV such that biological compositions can be quickly prepared for use as vaccines directed against the modified virus or its altered antigenic components.

SUMMARY OF THE INVENTION

Specific mutations in amino acid sequence are represented herein as "Xaa_1nXaa_2" where Xaa_1 is the original amino acid residue before mutation, n is the residue number and Xaa_2 is the mutant amino acid. The abbreviation "Xaa" may be the three letter or single letter amino acid code. A mutation in single letter code is represented, for example, by X_1nX_2 where X_1 and X_2 are the same as Xaa_1 and Xaa_2, respectively. The amino acid residues for Hepatitis B virus DNA polymerase are numbered with the residue methionine in the motif Tyr Met Asp Asp (YMDD) being residue number 550.

The reference HBV is considered herein to comprise a composite or consensus nucleotide or amino acid sequence from HBV genotypes A through F.

One aspect of the present invention provides a variant HBV comprising a surface component exhibiting an altered immunological profile compared to a reference HBV.

Another aspect of the present invention is directed to a variant HBV comprising a surface antigen having an amino acid sequence with a single or multiple amino acid substitution, addition and/or deletion or a truncation compared to a surface antigen from a reference HBV and wherein an antibody generated to the reference surface antigen exhibits reduced capacity for neutralizing said HBV variant.

Yet another aspect of the present invention provides an HBV variant comprising a surface antigen having an amino acid sequence with a single or multiple amino acid substitution, addition and/or deletion or truncation compared to the amino acid sequence set forth in Formula I and wherein the surface antigen of the variant HBV exhibits an altered

immunological profile compared to the surface antigen defined by Formula I and wherein the variant HBV is selected for by a nucleoside analogue of the HBV DNA polymerase.

Still another aspect of the present invention is directed to an HBV variant comprising a surface antigen having an amino acid sequence with a single or multiple amino acid substitution, addition and/or deletion or truncation compared to the amino acid sequence set forth in Formula I and wherein the surface antigen of the variant HBV exhibits an altered immunological profile compared to the surface antigen defined by Formula I and wherein the variant HBV is selected for following immunological therapy directed against the surface antigen as defined in Formula I.

Even still another aspect of the present invention provides an HBV variant comprising a nucleotide sequence comprising a single or multiple nucleotide substitution, addition and/or deletion to the nucleotide sequence set forth in Formula III and which HBV variant has a surface antigen exhibiting an altered immunological profile relative to a surface antigen defined by Formula I.

Another aspect of the present invention provides an isolated HBsAg or a recombinant form thereof or derivative or chemical equivalent thereof.

Yet another aspect of the present invention is directed to an isolated variant HBsAg or a recombinant or derivative form thereof or a chemical equivalent thereof wherein said HBsAg or its recombinant or derivative form or its chemical equivalent exhibits an altered immunological profile compared to an HBsAg from a reference HBV.

Still yet another aspect of the present invention provides an isolated variant HBsAg or a recombinant or derivative form thereof or a chemical equivalent thereof wherein said HBsAg or its recombinant or derivative form or its chemical equivalent comprises an amino acid sequence with a single or multiple amino acid substitution, addition and/or deletion or a truncation compared to an HBsAg from a reference HBV and wherein a

neutralising antibody directed to a reference HBV exhibits no or reduced neutralising activity to an HBV carrying said variant HBsAg.

Another aspect of the present invention contemplates a biological composition comprising a variant HBV or an HBsAg from said variant HBV or a recombinant or derivative form thereof or its chemical equivalent.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a diagrammatic representation showing overlapping genome of HBV.

Figure 2 is a representation of the amino acid consensus sequence from HBV DNA polymerase proteins encompassing regions which are conserved in the RNA polymerase protein. These regions are shown as domains A-E and are underlined. In the consensus sequence the M in the YMDD motif is designated as amino acid number 550. The amino acids which are subject to mutation during 3TC and/or FCV treatment are shown in bold. An asterisk (*) indicates greater than three amino acid possibilities at this position of the consensus sequence. The HBsAg major hydrophilic region containing the neutralisation domain is indicated by a double line and the polymerase mutations which alter the HBsAg are indicated in italics.

Figure 3 is a representation of the nucleotide sequence from various strains of HBV encoding the surface antigen. The amino acid sequence of the surface antigen beginning at amino acid 108 is shown above the nucleotide sequence.

Figure 4 is a graphical representation showing HBsAg binding assay with wild-type (i.e. reference HBV) and various mutants (1, mock; 2; wild-type; 3, F512L; 4, V519L; 5, M550I; 6, S565P; 7, double mutant L256M + M550V; 8, triple mutant V519L + L526M + M550V; 9, W499Q).

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is predicated in part on the identification of HBV variants exhibiting an altered immunological profile with respect to a surface component relative to a reference standard. The term "variant" is used in its broadest context and includes mutants such as vaccine escape mutants, derivatives, modified forms of and altered forms of an HBV relative to a reference HBV. A variant generally contains a single or multiple nucleotide substitution, addition and/or deletion or a truncation mutation in the viral genome and a corresponding single or multiple amino acid substitution, addition and/or deletion or truncation in a viral peptide, polypeptide or protein.

A preferred variant in accordance with the present invention with an altered immunological profile is one which would substantially not be affected by a neutralizing immune response directed to a conventional HBV vaccine such as a vaccine comprising a reference HBV or a surface component thereof. The expression "substantially not affected" includes reduced susceptibility to the immune response generated by a vaccine. Reduced susceptibility may also be conveniently determined by reduced susceptibility to chemical agents such as nucleoside analogues which target HBV DNA polymerase. Due to the overlapping nature of reading frames for DNA polymerase and certain viral surface components, an altered surface component may have a corresponding alteration in the DNA polymerase.

The preferred surface component of the HBV of the present invention is the HBV surface antigen (HBsAg). It is proposed in accordance with the present invention that the HBsAg of the HBV variants exhibit an altered immune profile relative to an HBsAg from a reference HBV. For the purposes of the present invention, a reference HBV conveniently comprises an HBsAg with an amino acid sequence substantially as set forth by Norder *et al.* (9) which encompasses all known genotypes of HBV (currently A through F). The amino acid sequence of an HBsAg and which is considered to define a reference HBV is set forth below in Formula I:

FORMULA I

M X_1 X_2 X_3 X_4 S G X_5 L X_6 P L X_7 V L Q A X_8 X_9 F X_{10} L T X_{11} I X_{12} X_{13} I P X_{14} S L X_{15} S W W T S L N F L G X_{16} X_{17} X_{18} X_{19} C X_{20} G X_{21} N X_{22} Q S X_{23} X_{24} S X_{25} H X_{26} P X_{27} X_{28} C P P X_{29} C X_{30} G Y R W M C L X_{31} R F I I F L X_{32} I L L L C L I F L L V L L D X_{33} Q G M L X_{34} V C P L X_{35} P X_{36} X_{37} X_{38} T T S X_{39} X_{40} X_{41} C X_{42} T C X_{43} X_{44} X_{45} X_{46} Q G X_{47} S X_{48} X_{49} P X_{50} X_{51} C C X_{52} K P X_{53} X_{54} G N C T C I P I P S X_{55} W A X_{56} X_{57} X_{58} X_{59} L W E X_{60} X_{61} S X_{62} R X_{63} S W L X_{64}LL$X_{65}$$X_{66}$ F V Q X_{67} X_{68} X_{69} X_{70} L X_{71} P X_{72} V W X_{73} X_{74} X_{75} I W X_{76} X_{77} W X_{78} W X_{79} P X_{80} X_{81} X_{82} X_{83} I X_{84} X_{85} P F X_{86} P L L P I F X_{87} X_{88} L X_{89} X_{90} X_{91} I

wherein:

X_1 is E or G or D;

X_2 is N or S or K;

X_3 is I or T;

X_4 is T or A;

X_5 is F or L;

X_6 is G or R;

X_7 is L or R;

X_8 is G or V;

X_9 is F or C;

X_{10} is L or S or W;

X_{11} is R or K;

X_{12} is L or R;

X_{13} is T or K;

X_{14} is Q or K;

X_{15} is D or H;

X_{16} is G or E or A;

X_{17} is S or A or V or T or L;

X_{18} is P or T;

X_{19} is V or R or T or K or G;

X_{20} is L or P;

X_{21} is Q or L or K;

X_{22} is S or L;

X_{23} is P or Q;

X_{24} is T or I;

X_{25} is N or S;

X_{26} is S or L;

X_{27} is T or I;

X_{28} is S or C;

X_{29} is I or T;

X_{30} is P or A;

X_{31} is R or Q;

X_{32} is F or C;

X_{33} is Y or C;

X_{34} is P or H or S;

X_{35} is I or L;

X_{36} is G or R;

X_{37} is S or T;

X_{38} is T or S;

X_{39} is T or V or A;

X_{40} is G or E or Q;

X_{41} is P or A or S;

X_{42} is K or R;

X_{43} is T or M;

X_{44} is T or I or S or A;

X_{45} is P or T or A or I or L;

X_{46} is A or V;

X_{47} is N or T;

X_{48} is M or K or L;

X_{49} is F or Y or I;

X_{50} is S or Y;

X_{51} is C or S;

X_{52} is T or I or S;

X_{53} is T or S;

X_{54} is D or A;

X_{55} is S or T;

X_{56} is F or L;

X_{57} is A or G or V;

X_{58} is K or R or T;

X_{59} is Y or F;

X_{60} is W or G;

X_{61} is A or G;

X_{62} is V or A;

X_{63} is F or L;

X_{64} is S or N;

X_{65} is V or A;

X_{66} is P or Q;

X_{67} is W or C or S;

X_{68} is F or C;

X_{69} is V or D or A;

X_{70} is G or E;

X_{71} is S or F;

X_{72} is T or I;

X_{73} is L or P;

X_{74} is S or L;

X_{75} is A or V;

X_{76} is M or I;

X_{77} is M or I;

X_{78} is Y or F;

X_{79} is G or E;

X_{80} is S or N or K;

X_{81} is L or Q;

X_{82} is Y or F or H or C;

X_{83} is S or G or N or D or T;

X_{84} is V or L;

X_{85} is S or N;

X_{86} is I or M or L;

X_{87} is F or C;

X_{88} is C or Y;

X_{89} is W or R;

X_{90} is V or A; and

X_{91} is Y or I or S.

Accordingly, one aspect of the present invention provides a variant HBV comprising a surface component exhibiting an altered immunological profile compared to a reference HBV.

More particularly, the present invention is directed to a variant HBV comprising a surface antigen having an amino acid sequence with a single or multiple amino acid substitution, addition and/or deletion or a truncation compared to a surface antigen from a reference HBV and wherein an antibody generated to the reference surface antigen exhibits reduced capacity for neutralizing said HBV variant.

The amino acid sequence of the HBsAg of the reference HBV is as set forth in Formula I above.

The HBV variant of the present invention is also referred to herein as an "escape" mutant since it is substantially incapable of being adversely effected by chemical therapy directed against the HBV polymerase or vaccine therapy directed against the surface antigen. The term "escape" mutant also encompasses reduced susceptibility to chemical or vaccine therapy directed to the reference HBV.

The HBV variant of the present invention is also preferably in isolated form. An isolated HBV includes reference to a biologically pure form of the virus. The term "isolated" means the virus has undergone at least one purification or isolation step away from non-viral components. Preferably, the viral preparation comprises at least about 10%, more preferably at least about 20%, still more preferably at least about 30%, even more preferably at least about 40%, yet more preferably at least about 50% or greater of HBV variant relative to the non-viral components as measured by viral infectivity, immunological interactivity, DNA polymerase activity, molecular weight, carbohydrate content or other suitable means.

The preferred variants of the present invention are obtained following selective pressure. The preferred selective pressure is chemical pressure (e.g. *via* nucleoside analogues) directed to the HBV DNA polymerase which selects for a mutation in the gene encoding HBV DNA polymerase and a corresponding mutation in the gene encoding HBsAg. This is due to the overlapping open reading frames for HBV DNA polymerase and HBsAg. A

mutation in one or more nucleotides encoding HBV DNA polymerase may have an effect on the nucleotide sequence encoding HBsAg. The present invention also extends to changes in the HBsAg following immunological selection based on vaccines comprising HBsAg or a derivative thereof or an HBV comprising same and wherein the HBsAg comprises an amino acid sequence substantially as set forth in Formula I.

Accordingly, another aspect of the present invention provides an HBV variant comprising a surface antigen having an amino acid sequence with a single or multiple amino acid substitution, addition and/or deletion or truncation compared to the amino acid sequence set forth in Formula I and wherein the surface antigen of the variant HBV exhibits an altered immunological profile compared to the surface antigen defined by Formula I and wherein the variant HBV is selected for by a nucleoside analogue of the HBV DNA polymerase.

In a related embodiment the present invention is directed to an HBV variant comprising a surface antigen having an amino acid sequence with a single or multiple amino acid substitution, addition and/or deletion or truncation compared to the amino acid sequence set forth in Formula I and wherein the surface antigen of the variant HBV exhibits an altered immunological profile compared to the surface antigen defined by Formula I and wherein the variant HBV is selected for following immunological therapy directed against the surface antigen as defined in Formula I.

Reference to an altered immunological profile in accordance with the present invention in relation to the surface antigen includes reference to an altered humoral or T cell response. Examples of an altered immunological profile include altered specificity to antibodies, altered amino acid sequences of an epitope or within the "a" determinant, an altered capacity to induce proliferation of T cells primed to an HBsAg from a reference HBV. Preferably, the altered immunological profile means that neutralising antibodies which are capable of substantially neutralising or otherwise reducing serum or blood levels of the reference HBV are substantially incapable of or exhibit reduced capacity to neutralise

and/or clear the variant HBV.

A viral variant may, in accordance with a preferred aspect of the present invention, carry a mutation only in the DNA polymerase or the surface antigen or may carry a mutation in both molecules. The term "mutation" is to be read in its broadest context and includes a silent mutation not substantially affecting the normal function of the DNA polymerase or surface antigen or may be an active mutation having the effect of selection of nucleoside analogue resistance or a vaccine escape mutant phenotype. Where multiple mutations occur in accordance with the present invention or where multiple phenotypes result from a single mutation, at least one mutation must be active or the virus must exhibit at least one altered phenotype such as nucleoside analogue resistance or reduced immunological interactivity to the surface antigen of a reference HBV.

The present invention extends to any novel mutant or novel use of a mutant of the HBsAg carrying a single or multiple substitution, addition and/or deletion or truncation in the amino acid sequence of HBsAg as compared to the amino acid sequence set forth in Formula I. In an alternative yet related embodiment, the present invention extends to any single or multiple amino acid substitution, addition and/or deletion or truncation in the amino acid sequence of HBsAg relative to the amino acid sequence set forth in Formula I as defined by a single or multiple amino acid substitution, addition and/or deletion to the catalytic region of the HBV DNA polymerase set forth below in Formula II:

FORMULA II

S Z_1 L S W L S L D V S A A F Y H Z_2 P L H P A A M P H L L Z_3 G S S G L Z_4 R Y V A R L S S Z_5 S Z_6 Z_7 X N Z_8 Q Z_9 Z_{10} X X X Z_{11} L H Z_{12} Z_{13} C S R Z_{14} L Y V S L Z_{15} L L Y Z_{16} T Z_{17} G Z_{18} K L H L Z_{19} Z_{20} H P I Z_{21} L G F R K Z_{22} P M G Z_{23} G L S P F L L A Q F T S A I Z_{24} Z_{25} Z_{26} Z_{27} Z_{28} R A F Z_{29} H C Z_{30} Z_{31} F Z_{32} Y M* D D Z_{33} V L G A Z_{34} Z_{35} Z_{36} Z_{37} H Z_{38} E Z_{39} L Z_{40} Z_{41}

Z_{42} Z_{43} Z_{44} Z_{45} Z_{46} L L Z_{47} Z_{48} G I H L N P Z_{49} K T K R W G Y S L N F M G Y Z_{50} I G

wherein:

X is any amino acid;

Z_1 is N or D;

Z_2 is I or P;

Z_3 is I or V;

Z_4 is S or D;

Z_5 is T or N;

Z_6 is R or N;

Z_7 is N or I;

Z_8 is N or Y or H;

Z_9 is H or Y;

Z_{10} is G or R;

Z_{11} is D or N;

Z_{12} is D or N;

Z_{13} is S or Y;

Z_{14} is N or Q;

Z_{15} is L or M;

Z_{16} is K or Q;

Z_{17} is Y or F;

Z_{18} is R or W;

Z_{19} is Y or L;

Z_{20} is S or A;

Z_{21} is I or V;

Z_{22} is I or L;

Z_{23} is V or G;

Z_{24} is C or L;

Z_{25} is A or S;

Z_{26} is V or M;

Z_{27} is V or T;

Z_{28} is R or C;

Z_{29} is F or P;

Z_{30} is L or V;

Z_{31} is A or V;

Z_{32} is S or A;

Z_{33} is V or L or M;

Z_{34} is K or R;

Z_{35} is S or T;

Z_{36} is V or G;

Z_{37} is Q or E;

Z_{38} is L or S or R;

Z_{39} is S or F;

Z_{40} is F or Y;

Z_{41} is T or A;

Z_{42} is A or S;

Z_{43} is V or I;

Z_{44} is T or C;

Z_{45} is N or S;

Z_{46} is F or V;

Z_{47} is S or D;

Z_{48} is L or V;

Z_{49} is N or Q;

Z_{50} is V or I; and

M^{*} is amino acid 550.

Preferred mutations in the amino acid sequence of HBsAg are amino acid substitutions, deletions and/or additions or truncations in amino acids 1-10, 5-15, 10-20, 15-25, 20-30, 25-35, 30-40, 35-45, 40-50, 45-55, 50-60, 55-65, 60-70, 65-75, 70-80, 75-85, 80-90, 85-95, 90-100, 95-105, 100-110, 105-115, 110-120, 115-125, 120-130, 125-135, 130-140, 135-145, 140-150, 145-155, 150-160, 155-165, 160-170, 165-175, 170-180, 175-185, 180-190, 185-195, 190-200, 195-205, 200-210, 205-215, 210-220, 215-225, 220-226 (referring to the numbering of Formula I) of HBsAg. Particularly useful mutations are G112R, T123P Y/F134S, D144E, G145R, A157D, E164D, F170L, M195I, W196L, S196W, W196 STOP, M198I, W199S, S204T and S210R. The term "stop" means a stop codon.

Even more preferred mutations are D144E, G145R, A157D, E164D, M195I, W196L, S196W, W196 STOP, M198I, W199S and S210R.

The HBsAg mutations of the present invention may also be defined in terms of a corresponding mutation in the HBV DNA polymerase. A mutation in the HBV DNA polymerase may be in amino acids 421-431, 426-436, 431-441, 436-446, 441-451, 446-456, 451-461, 456-466, 461-471, 466-476, 471-481, 476-486, 481-491, 486-496, 491-501, 496-506, 501-511, 506-516, 511-521, 516-526, 521-531, 526-536, 531-541, 536-546, 541-551, 546-556, 551-561, 556-566, 561-571, 566-576, 571-581, 576-586, 581-591, 586-596, 591-601, 596-601 (referring to number of Formula II).

Preferred HBV DNA polymerase mutations include Q476, N480G, N485K, K495R, R499O, G499E, W499Q, FJ12L, I515L, V519L, L526M, M550V, M550I, V553I, S565P. Useful multiple mutants include L526M/M550I, L526M/M550V, V519L/L526M/M550V and V519L/L526M/M550I.

The altered HBsAg molecules of the HBV variants of the present invention may also be

defined at the nucleotide level. The nucleotide sequence encoding the HBsAg from a reference HBV is set forth below in Formula III:

FORMULA III

A C N_1 A A A C C T N_2 N_3 G G A N_4 G G A A A N_5 T G C A C N_6 T G T A T T C C C A T C C C A T C N_7 T C N_8 T G G G C T T T C G N_9 A A N_{10} A T N_{11} C C T A T G G G A G N_{12} G G G C C T C A G N_{13} C C G T T T C T C N_{14} T G G C T C A G T T T A C T A G T G C C A T T T G T T C A G T G G T T C G N_{15} A G G G C T T T C C C C C A C T G T N_{16} T G G C T T T C A G N_{17} T A T A T G G A T G A T G T G G T N_{18} T T G G G G G C C A A G T C T G T A C A N_{19} C A T C N_{20} T G A G T C C C T T T N_{21} T N_{22} C C N_{23} C T N_{24} T T A C C A A T T T T C T T N_{25} T G T C T N_{26} T G G G N_{27} A T A C A T T

wherein:

N_1 is A or C;

N_2 is T or A;

N_3 is C or T;

N_4 is C or T;

N_5 is C or T;

N_6 is C or T;

N_7 is A or G;

N_8 is T or C;

N_9 is C or G;

N_{10} is G or A;

N_{11} is T or A;

N_{12} is T or G;

N_{13} is T or C;
N_{14} is C or T;
N_{15} is T or C;
N_{16} is T or C;
N_{17} is T or C;
N_{18} is A or T;
N_{19} is A or G;
N_{20} is T or G;
N_{21} is A or T;
N_{22} is A or G;
N_{23} is T or G;
N_{24} is A or G;
N_{25} is T or C;
N_{26} is T or C; and
N_{27} is T or C.

The present invention extends to nucleotide sequences which exhibit at least about 60% nucleotide sequence identity to Formula III or is a sequence capable of hybridising thereto under low stringency conditions at 42 °C and which encode an HBsAg with an altered immunological profile relative to an HBsAg from a reference HBV.

Accordingly, another aspect of the present invention provides an HBV variant comprising a nucleotide sequence comprising a single or multiple nucleotide substitution, addition and/or deletion to the nucleotide sequence set forth in Formula III and which HBV variant has a surface antigen exhibiting an altered immunological profile relative to a surface antigen defined by Formula I.

Preferably, the HBV variant comprises a nucleotide sequence having at least about 80% identity to the nucleotide sequence set forth in Formula III or is capable of hybridising

thereto under medium stringency conditions at 42 °C. Preferably, the percentage identity is at least about 85%, at least about 90%, at least about 95%, but less than 100% relative to the nucleotide sequence set forth in Formula III.

The term "similarity" as used herein includes exact identity between compared sequences at the nucleotide or amino acid level. Where there is non-identity at the nucleotide level, "similarity" includes differences between sequences which result in different amino acids that are nevertheless related to each other at the structural, functional, biochemical and/or conformational levels. Where there is non-identity at the amino acid level, "similarity" includes amino acids that are nevertheless related to each other at the structural, functional, biochemical and/or conformational levels. In a particularly preferred embodiment, nucleotide and sequence comparisons are made at the level of identity rather than similarity. Any number of programs are available to compare nucleotide and amino acid sequences. Preferred programs have regard to an appropriate alignment. One such program is Gap which considers all possible alignment and gap positions and creates an alignment with the largest number of matched bases and the fewest gaps. Gap uses the alignment method of Needleman and Wunsch (10). Gap reads a scoring matrix that contains values for every possible GCG symbol match. GAP is available on ANGIS (Australian National Genomic Information Service) at website http://mel1.angis.org.au..

Reference herein to a low stringency at 42°C includes and encompasses from at least about 0% v/v to at least about 15% v/v formamide and from at least about 1M to at least about 2M salt for hybridisation, and at least about 1M to at least about 2M salt for washing conditions. Alternative stringency conditions may be applied where necessary, such as medium stringency, which includes and encompasses from at least about 16% v/v to at least about 30% v/v formamide and from at least about 0.5M to at least about 0.9M salt for hybridisation, and at least about 0.5M to at least about 0.9M salt for washing conditions, or high stringency, which includes and encompasses from at least about 31% v/v to at least about 50% v/v formamide and from at least about 0.01M to at least about 0.15M salt for hybridisation, and at least about 0.01M to at least about 0.15M salt for washing conditions. In general, washing is carried out $T_m = 69.3 + 0.41$ (G+C)% [11]. However, the T_m of a

duplex DNA decreases by 1°C with every increase of 1% in the number of mismatch base pairs (12).

The present invention further extends to an isolated surface component from the HBV variants herein described. More particularly, the present invention provides an isolated HBsAg or a recombinant form thereof or derivative or chemical equivalent thereof. The isolated surface component and, more particularly, isolated HBsAg or its recombinant, derivative or chemical equivalents are useful in the development of biological compositions such as vaccine formulations.

Accordingly, another aspect of the present invention is directed to an isolated variant HBsAg or a recombinant or derivative form thereof or a chemical equivalent thereof wherein said HBsAg or its recombinant or derivative form or its chemical equivalent exhibits an altered immunological profile compared to an HBsAg from a reference HBV.

More particularly, the present invention provides an isolated variant HBsAg or a recombinant or derivative form thereof or a chemical equivalent thereof wherein said HBsAg or its recombinant or derivative form or its chemical equivalent comprises an amino acid sequence with a single or multiple amino acid substitution, addition and/or deletion or a truncation compared to an HBsAg from a reference HBV and wherein a neutralising antibody directed to a reference HBV exhibits no or reduced neutralising activity to an HBV carrying said variant HBsAg.

The term "isolated" means the same as it does in relation to an isolated HBV variant.

The reference HBV is conveniently defined herein as comprising an HBsAg with an amino acid sequence as set forth in Formula I or as indirectly defined by the amino acid sequence for HBV DNA polymerase set forth in Formula II or by the nucleotide sequence set forth in Formula III encoding an HBsAg.

As stated above, the present invention extends to derivatives and chemical equivalents (i.e.

analogues) of the HBV surface component and in particular HBsAg. Derivatives include single or multiple amino acid substitutions, additions and/or deletions to the HBsAg molecule. "Additions" to amino acid sequences include fusions with other peptides, polypeptides or proteins or fusions to nucleotide sequences including fusions to other viral components.

Analogues of the variant HBsAg contemplated herein include, but are not limited to, modification to side chains, incorporating of unnatural amino acids and/or their derivatives during peptide, polypeptide or protein synthesis and the use of crosslinkers and other methods which impose conformational constraints on the proteinaceous molecule or their analogues. These types of modifications are useful in stabilizing the immunointeractive molecules for use in diagnostic assays or in therapeutic protocols.

Examples of side chain modifications contemplated by the present invention include modifications of amino groups such as by reductive alkylation by reaction with an aldehyde followed by reduction with $NaBH_4$; amidination with methylacetimidate; acylation with acetic anhydride; carbamoylation of amino groups with cyanate; trinitrobenzylation of amino groups with 2, 4, 6-trinitrobenzene sulphonic acid (TNBS); acylation of amino groups with succinic anhydride and tetrahydrophthalic anhydride; and pyridoxylation of lysine with pyridoxal-5-phosphate followed by reduction with $NaBH_4$.

The guanidine group of arginine residues may be modified by the formation of heterocyclic condensation products with reagents such as 2,3-butanedione, phenylglyoxal and glyoxal.

The carboxyl group may be modified by carbodiimide activation *via* O-acylisourea formation followed by subsequent derivitisation, for example, to a corresponding amide.

Sulphydryl groups may be modified by methods such as carboxymethylation with iodoacetic acid or iodoacetamide; performic acid oxidation to cysteic acid; formation of a mixed disulphides with other thiol compounds; reaction with maleimide, maleic anhydride or other substituted maleimide; formation of mercurial derivatives using 4-chloromercuribenzoate,

4-chloromercuriphenylsulphonic acid, phenylmercury chloride, 2-chloromercuri-4-nitrophenol and other mercurials; carbamoylation with cyanate at alkaline pH.

Tryptophan residues may be modified by, for example, oxidation with N-bromosuccinimide or alkylation of the indole ring with 2-hydroxy-5-nitrobenzyl bromide or sulphenyl halides. Tyrosine residues on the other hand, may be altered by nitration with tetranitromethane to form a 3-nitrotyrosine derivative.

Modification of the imidazole ring of a histidine residue may be accomplished by alkylation with iodoacetic acid derivatives or N-carbethoxylation with diethylpyrocarbonate.

Examples of incorporating unnatural amino acids and derivatives during peptide synthesis include, but are not limited to, use of norleucine, 4-amino butyric acid, 4-amino-3-hydroxy-5-phenylpentanoic acid, 6-aminohexanoic acid, t-butylglycine, norvaline, phenylglycine, ornithine, sarcosine, 4-amino-3-hydroxy-6-methylheptanoic acid, 2-thienyl alanine and/or D-isomers of amino acids. A list of unnatural amino acid, contemplated herein is shown below in Table 1. The inclusion of such unnatural amino acids or other derivations described herein may assist in stabilising the molecule in a vaccine composition.

TABLE 1

Non-conventional amino acid	Code	Non-conventional amino acid	Code
α-aminobutyric acid	Abu	L-N-methylalanine	Nmala
α-amino-α-methylbutyrate	Mgabu	L-N-methylarginine	Nmarg
aminocyclopropane-carboxylate	Cpro	L-N-methylasparagine	Nmasn
		L-N-methylaspartic acid	Nmasp
aminoisobutyric acid	Aib	L-N-methylcysteine	Nmcys
aminonorbornyl-carboxylate	Norb	L-N-methylglutamine	Nmgln
		L-N-methylglutamic acid	Nmglu
cyclohexylalanine		Chexa L-N-methylhistidine	Nmhis
cyclopentylalanine	Cpen	L-N-methylisolleucine	Nmile
D-alanine	Dal	L-N-methylleucine	Nmleu
D-arginine	Darg	L-N-methyllysine	Nmlys
D-aspartic acid	Dasp	L-N-methylmethionine	Nmmet
D-cysteine	Dcys	L-N-methylnorleucine	Nmnle
D-glutamine	Dgln	L-N-methylnorvaline	Nmnva
D-glutamic acid	Dglu	L-N-methylornithine	Nmorn
D-histidine	Dhis	L-N-methylphenylalanine	Nmphe
D-isoleucine	Dile	L-N-methylproline	Nmpro
D-leucine	Dleu	L-N-methylserine	Nmser
D-lysine	Dlys	L-N-methylthreonine	Nmthr
D-methionine	Dmet	L-N-methyltryptophan	Nmtrp
D-ornithine	Dorn	L-N-methyltyrosine	Nmtyr
D-phenylalanine	Dphe	L-N-methylvaline	Nmval
D-proline	Dpro	L-N-methylethylglycine	Nmetg
D-serine	Dser	L-N-methyl-t-butylglycine	Nmtbug
D-threonine	Dthr	L-norleucine	Nle
D-tryptophan	Dtrp	L-norvaline	Nva

D-tyrosine	Dtyr	α-methyl-aminoisobutyrate	Maib
D-valine	Dval	α-methyl-γ-aminobutyrate	Mgabu
D-α-methylalanine	Dmala	α-methylcyclohexylalanine	Mchexa
D-α-methylarginine	Dmarg	α-methylcylcopentylalanine	Mcpen
D-α-methylasparagine	Dmasn	α-methyl-α-napthylalanine	Manap
D-α-methylaspartate	Dmasp	α-methylpenicillamine	Mpen
D-α-methylcysteine	Dmcys	N-(4-aminobutyl)glycine	Nglu
D-α-methylglutamine	Dmgln	N-(2-aminoethyl)glycine	Naeg
D-α-methylhistidine	Dmhis	N-(3-aminopropyl)glycine	Norn
D-α-methylisoleucine	Dmile	N-amino-α-methylbutyrate	Nmaabu
D-α-methylleucine	Dmleu	α-napthylalanine	Anap
D-α-methyllysine	Dmlys	N-benzylglycine	Nphe
D-α-methylmethionine	Dmmet	N-(2-carbamylethyl)glycine	Ngln
D-α-methylornithine	Dmorn	N-(carbamylmethyl)glycine	Nasn
D-α-methylphenylalanine	Dmphe	N-(2-carboxyethyl)glycine	Nglu
D-α-methylproline	Dmpro	N-(carboxymethyl)glycine	Nasp
D-α-methylserine	Dmser	N-cyclobutylglycine	Ncbut
D-α-methylthreonine	Dmthr	N-cycloheptylglycine	Nchep
D-α-methyltryptophan	Dmtrp	N-cyclohexylglycine	Nchex
D-α-methyltyrosine	Dmty	N-cyclodecylglycine	Ncdec
D-α-methylvaline	Dmval	N-cylcododecylglycine	Ncdod
D-N-methylalanine	Dnmala	N-cyclooctylglycine	Ncoct
D-N-methylarginine	Dnmarg	N-cyclopropylglycine	Ncpro
D-N-methylasparagine	Dnmasn	N-cycloundecylglycine	Ncund
D-N-methylaspartate	Dnmasp	N-(2,2-diphenylethyl)glycine	Nbhm
D-N-methylcysteine	Dnmcys	N-(3,3-diphenylpropyl)glycine	Nbhe
D-N-methylglutamine	Dnmgln	N-(3-guanidinopropyl)glycine	Narg
D-N-methylglutamate	Dnmglu	N-(1-hydroxyethyl)glycine	Nthr
D-N-methylhistidine	Dnmhis	N-(hydroxyethyl))glycine	Nser
D-N-methylisoleucine	Dnmile	N-(imidazolylethyl))glycine	Nhis
D-N-methylleucine	Dnmleu	N-(3-indolylyethyl)glycine	Nhtrp

	D-N-methyllysine	Dnmlys	N-methyl-γ-aminobutyrate	Nmgabu
	N-methylcyclohexylalanine	Nmchexa	D-N-methylmethionine	Dnmmet
	D-N-methylornithine	Dnmorn	N-methylcyclopentylalanine	Nmcpen
	N-methylglycine	Nala	D-N-methylphenylalanine	Dnmphe
5	N-methylaminoisobutyrate	Nmaib	D-N-methylproline	Dnmpro
	N-(1-methylpropyl)glycine	Nile	D-N-methylserine	Dnmser
	N-(2-methylpropyl)glycine	Nleu	D-N-methylthreonine	Dnmthr
	D-N-methyltryptophan	Dnmtrp	N-(1-methylethyl)glycine	Nval
	D-N-methyltyrosine	Dnmtyr	N-methyla-napthylalanine	Nmanap
10	D-N-methylvaline	Dnmval	N-methylpenicillamine	Nmpen
	γ-aminobutyric acid	Gabu	N-(*p*-hydroxyphenyl)glycine	Nhtyr
	L-*t*-butylglycine	Tbug	N-(thiomethyl)glycine	Ncys
	L-ethylglycine	Etg	penicillamine	Pen
	L-homophenylalanine	Hphe	L-α-methylalanine	Mala
15	L-α-methylarginine	Marg	L-α-methylasparagine	Masn
	L-α-methylaspartate	Masp	L-α-methyl-*t*-butylglycine	Mtbug
	L-α-methylcysteine	Mcys	L-methylethylglycine	Metg
	L-α-methylglutamine	Mgln	L-α-methylglutamate	Mglu
	L-α-methylhistidine	Mhis	L-α-methylhomophenylalanine	Mhphe
20	L-α-methylisoleucine	Mile	N-(2-methylthioethyl)glycine	Nmet
	L-α-methylleucine	Mleu	L-α-methyllysine	Mlys
	L-α-methylmethionine	Mmet	L-α-methylnorleucine	Mnle
	L-α-methylnorvaline	Mnva	L-α-methylornithine	Morn
	L-α-methylphenylalanine	Mphe	L-α-methylproline	Mpro
25	L-α-methylserine	Mser	L-α-methylthreonine	Mthr
	L-α-methyltryptophan	Mtrp	L-α-methyltyrosine	Mtyr
	L-α-methylvaline	Mval	L-N-methylhomophenylalanine	Nmhphe

N-(N-(2,2-diphenylethyl) carbamylmethyl)glycine	Nnbhm	N-(N-(3,3-diphenylpropyl) carbamylmethyl)glycine	Nnbhe
1-carboxy-1-(2,2-diphenyl-ethylamino)cyclopropane	Nmbc		

Crosslinkers can be used, for example, to stabilise 3D conformations, using homo-bifunctional crosslinkers such as the bifunctional imido esters having $(CH_2)_n$ spacer groups with n=1 to n=6, glutaraldehyde, N-hydroxysuccinimide esters and hetero-bifunctional reagents which usually contain an amino-reactive moiety such as N-hydroxysuccinimide and another group specific-reactive moiety such as maleimido or dithio moiety (SH) or carbodiimide (COOH). In addition, peptides can be conformationally constrained by, for example, incorporation of C_α and N_α-methylamino acids, introduction of double bonds between C_α and C_β atoms of amino acids and the formation of cyclic peptides or analogues by introducing covalent bonds such as forming an amide bond between the N and C termini, between two side chains or between a side chain and the N or C terminus.

As stated above, these types of modifications may be important to stabilise the variant HBsAg molecule if administered to an individual or for use as a diagnostic reagent.

Other derivatives contemplated by the present invention include a range of glycosylation variants from a completely unglycosylated molecule to a modified glycosylated molecule. Altered glycosylation patterns may result from expression of recombinant molecules in different host cells.

Another aspect of the present invention extends to the variant HBsAg molecule or its recombinant, derivative or chemical form or a variant HBV comprising said HBsAg in composition form. Such compositions are particularly useful as therapeutic compositions and may be referred to herein interchangeably as biological, vaccine or pharmaceutical compositions. The biological compositions are particularly useful in inducing immunological memory against infection by an HBV variant such as an HBV escape

mutant controlling by administering a variant HBsAg or a recombinant, derivative or chemical form thereof or an HBV comprising same capable of inducing an immune response including immunological memory agents.

Accordingly, the present invention contemplates a biological composition comprising a variant HBV or an HBsAg from said variant HBV or a recombinant or derivative form thereof or its chemical equivalent.

Generally, if an HBV is used, it is first attenuated. The biological composition according to this aspect of the present invention generally further comprises one or more pharmaceutically acceptable carriers and/or diluents.

The biological composition may comprise an HBsAg or like molecule from one HBV variant or the composition may be a cocktail of HBsAgs or like molecules from a range of HBV variants including the referenced HBV. Similar inclusions apply where the composition comprises an HBV.

The biological composition forms suitable for injectable use include sterile aqueous solutions (where water soluble) or sterile powders for the extemporaneous preparation of sterile injectable solutions. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or diluent containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The preventions of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the HBsAg or like molecule or HBV variant or reference strain in the required amount in the appropriate solvent or diluent as followed by sterilization such as by filter sterilization. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and the freeze-drying technique which yield a powder of the immunointeractive molecule plus any additional desired ingredient from previously sterile-filtered solution thereof. Routes of administration contemplated by the present invention including intravenous, intraperitoneal, intrathelial, subcutaneous and intracerebral.

The biological composition of the present invention may also be given in oral, bucal, nasal spray, inhalation, patch, drip or suppository form.

Pharmaceutically acceptable carriers and/or diluents include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the immunointeractive molecule, use thereof in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

The HBsAg or like molecule or HBV variant or reference strain will be added in a concentration effective to induce an interact immune response against the same molecule or an HBV carrying the same or an immunologically similar molecule. For example, an effective amount of HBsAg may range from about 10 mg to about 2000 ng, or 50 ng to about 1000 mg or 100 ng to about 500 mg or other suitable effective amount. It is sometimes more convenient to express dosage amounts in terms of body weight. Accordingly, the effective amounts may be from, for example, about 0.5 ng/kg body weight to about 500 mg/kg body weight or an amount there between.

The present invention is further described by the following non-limiting Examples.

EXAMPLE 1

OVERLAPPING GENOME OF HBV

The overlapping genome of HBV is represented in Figure 1. The gene encoding DNA polymerase (P), overlaps the viral envelope genes, Pre-S1 and Pre-S2, and partially overlaps the X and core (C) genes. The HBV envelope comprises small, middle and large HBV surface antigens. The large protein component is referred to as the HBV surface antigen (HBsAg) and is enclosed by the S gene sequence. The Pre-S1 and Pre-S2 gene sequences encode the other envelope components.

EXAMPLE 2

AMINO ACID CONSENSUS SEQUENCE FO HBV DNA POLYMERASE

The amino acid consensus sequence for HBV DNA polymerase protein from genotypes A through F is shown in Figure 2.

EXAMPLE 3

CONSENSUS SEQUENCE OF HBsAg

The nucleotide sequence from various strains of HBV encoding the surface antigen is shown in Figure 3. The amino acid sequence of the surface antigen beginning at amino acid 108 is shown above the nucleotide sequence.

EXAMPLE 4

HBsAg BINDING ASSAY

The effect of the Pre-S/S gene escape mutations on the binding of anti-HBs antibody is assessed using an RIA binding assay. The results are shown in Figure 4. Briefly, the expressed mutant HBsAg from transfected cell cultures is purified through a sucrose density gradient. The ability of subviral and viral particles to block the binding of wild type HBsAg to anti-HBs antibody, which does not recognise S gene escape mutants, is assessed

in an RIA format (AUSAB, Abbott). This analysis involves the binding of anti-HBs in pooled vaccine serum to increasing concentrations of wild type and mutant S protein using limiting concentrations of serum and detecting the unbound anti-HBs by AUSAB RIA.

The mutant S proteins analysed are shown on the right of Figure 4 together with mock and wild type HBV. As the concentration of HBsAg decreases the amount of unbound anti-HBs increase, leaving a higher anti-HBs concentration to be detected by the AUSAB assay. Even at high concentration of HBsAg from the W499Q mutant the amount of residual anti-HBs detected is similar to that of the mock transfected sample (these are represented by the two curves at the top of the graph). In contrast, the amount of residual anti-HBs after binding of antibody with the other mutant HBsAg proteins is analogous to the wild type HBsAg, indicating that these variant vHBsAg proteins recognise the anti-HBs with similar efficiency as the wild type protein.

Two of the mutant S proteins (Figure 4: V519L and the triple mutant which contains the mutations V519L + L526M +M550V with respect to the polymerase protein in the overlapping reading frame) had partial binding of anti-HBsAg. The binding efficacy of the mutant S proteins to HBsAg is altered when compared to wild type HBsAg. This suggests that viruses carrying these mutations may not be detected by anti-HBsAg as efficiently as wild type virus and thus may escape immune detection. Hepatitis B virus with these and/or other HBsAg mutations, which have partial binding to anti-HBsAg, may also escape immune detection and protection.

The dual mutant in Figure 4 represents L526M/M550V while the triple mutant represents V519L/L526M/M550V.

EXAMPLE 5

HBV VARIANTS PRODUCED BY SITE DIRECTED MUTAGENESIS

Table 2 provides a summary of some of the HBV variants produced by site directed mutagenesis.

EXAMPLE 6
FCV MUTATION

Table 3 provides a summary of mutations induced by famciclovir (FCV).

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

TABLE 2

HBV VARIANTS PRODUCED BY SITE DIRECTED MUTAGENESIS

Nucleotide analogue selected	
Polymerase mutations	**Corresponding surface S mutation**
1. G499E (B domain)	D144E
2. W499Q (B domain)	G145R
3. F512L (B domain)	A157D
4. V519L (B domain)	E164D
5. L526M (B domain)	no change
6. M550V (C domain)	M195I
7. M550V (C domain)	W196L
8. M550I (C domain)	S196W
9. V553I (C domain)	M198I
10. V553I (C domain)	W199S
11. S565P	S210R
Double polymerase mutation	**Corresponding S mutation**
12. L526M/M550V	M195I
13. L526M/M550I	S196W
Triple polymerase mutation	**Corresponding S mutation**
14. V519L/L526M/M550V	E164D, M195I, W196L
15. VS19L/L526M/M550I	E164D, S196W
HBsAg escape mutant	**Corresponding HBV polymerase changes**
16. K122R (loop 1 "a" determinant)	Q476P
17. T126S (loop 1 "a" determinant)	N480G
18. T131N (loop 1 "a" determinant)	N485K
19. K141E (loop 2 "a" determinant)	K495R
20. G145K (loop 2 "a" determinant)	R499Q
21. R160N	I515L

TABLE 3

FCV Mutations		Number of patients with Mutation (%)	
S421L	A Domain	1/34	3%
N422K	A Domain	1/34	3%
L423L/M/V	A Domain	1/34	3%
S424T	A Domain	2/34	6%
S/D 455P		7/34*	20.5%
N464D		1/34	3%
Q471K/N		2/34	6%
D/N 480E		1/34	3%
T484H		1/34	3%
R499K		4/34	12%
V519L	B Domain	3/34	9%
L/M/V523L	B Domain	1/34	3%
F524L/F	B Domain	1/34	3%
L526M	B Domain	5/34	15%
A527T	B Domain	1/34	3%
I533I/V		2/34	6%
V537I		1/34	3%
S565A		1/34	3%
S/D576F/S	D Domain	1/34	3%
L593V		1/34	3%
H/Y594H	E Domain	1/34	3%
T/M596M	E Domain	1/34	3%

* Only detected in BMT patients on FCV

BIBLIOGRAPHY:

1. Tiollais *et al. Nature 317:* 489-495, 1985.

2. Gerlich *et al. Viral Hepatitis and Liver Disease.* F.B. Hollinger *et al.* eds Williams-Wilkens, Baltimore, MD, pp121-134, 1991.

3. Carman *et al. Gastroenterology 102:.* 711-719, 1992.

4. Carman *et al. Lancet 336:* 325-329, 1990.

5. Okamoto *et al. Paediatric Research 32:* 264-268, 1992.

6. McMahon *et al. Hepatology 15:* 757-766, 1992.

7. Fujii *et al. Biochem. Biophys. Res. Commun. 184:* 1152-1157, 1992.

8. Harrison *et al. J. Hepatol. 13:* 5105-5107, 1991.

9. Norder *et al. J. Gen. Virol. 74:* 1341-1348, 1993.

10. Needleman and Wunsch *J. Mol. Biol. 48:* 443-453, 1970.

11. Marmur and Doty *J. Mol. Biol. 5:* 109, 1962.

12. Bonner and Laskey *Eur. J. Biochem. 46:* 83, 1974.